TITLE: ATM and DNA-PKcs make a complementary couple in DNA double strand break repair

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Abstract

The interplay between ATM and DNA-PKcs kinases during double strand breaks (DSBs) resolution is still a matter of debate. ATM and DNA-PKcs participate differently in the DNA damage response pathway (DDR), but important common aspects are indeed found: both of them are activated when faced with DSBs, they share common targets in the DDR and the absence of either kinase results in faulty DSB repair. Absence of ATM translates into timely repair that, nevertheless, is incomplete. On the other hand, DNA-PKcs absence translates into slower repair, which in turn gives rise to the accumulation of simple and complex reorganizations. These outcomes confirm that the function of both protein kinases is essential to guarantee genome integrity. Interestingly, V(D)J and CSR recombination events provide a powerful tool to study the interplay between both kinases in DSB repair. Although the physiological DSBs generated during V(D)J and CSR recombination are resolved by the non-homologous end-joining (NHEJ) repair pathway, ATM absence during these events translates into chromosome translocations. These results suggest that NHEJ accuracy is threatened in the absence of ATM, which may play a role in avoiding illegitimate repair by favouring the joining of the correct DNA ends. Indeed, simultaneous DNA-PKcs and ATM deficiency during V(D)J and CSR recombination translates into a synergistic increase in potentially dangerous chromosomal translocations and deletions. Although the exact nature of their interaction remains elusive, the evidence indicates that ATM and DNA-PKcs play complementary roles that allow complete and legitimate DSB repair to be reached. Faithful repair can only be achieved by the presence and correct functioning of both kinases: while DNA-PKcs ensures fast rejoining, ATM guarantees complete repair.

DSB generation and relevance: alerting to the presence of DSBs

Double strand breaks (DSBs) can be generated during multiple physiological processes such as meiotic, class switch (CSR) or V(D)J recombination events, but they can also result from various external and internal insults, such as radiation, radiomimetic drugs or oxygen free radicals arising from the cell metabolism. Unlike single strand breaks, an intact complementary strand is not available for DSB repair; thus, they are considered the most hazardous lesion the cell can suffer. When DSBs remain unrepaired they are generally referred to as residual DSBs, which are believed to be potentially lethal[1]. However, DSBs are generally rejoined. In vertebrate cells most DSBs are repaired by non-homologous end joining (NHEJ), which does not depend on the presence of an undamaged template, as well as homologous recombination (HR), which does require an undamaged DNA template to perform repair (for a review on these two repair pathways see [2,3]and[4]). Although both repair pathways can legitimately rejoin DSBs, the NHEJ machinery can frequently introduce short deletions or insertions at the joining site, even when the two original broken ends are ligated. In addition to this, and due to the absence of a DNA template, NHEJ can also join illegitimate DNA ends. Illegitimate joining can translate into chromosomal rearrangements, whose accumulation can give rise to the onset of genomic instability.

In order to effectively resolve DSBs with either of these repair pathways, the DSB must be efficiently signalled, and repair proteins must be recruited while the cell cycle is halted. These functions are performed by the DNA damage response (DDR) machinery. DNA-PKcs (the catalytic subunit of the DNA-dependent protein kinase) is a key component of the NHEJ pathway, although efficient DSB repair also requires the presence of DNA LigaseIV/XRCC4, Artemis, XRCC4-like factor (XLF) and Ku heterodimer. DNA-PKcs is a member of the phosphatidylinositol 3-kinase-related kinase family (PIKKs), which also includes ataxia-telangiectasia mutated (ATM) and ATM and Rad3 related (ATR). ATM is a central signal transducer in the DDR, and most DSB signal transduction pathways are thought to be governed cooperatively by ATM and ATR, as at least 700

proteins are phosphorylated on ATM/ATR consensus sites in response to radiation[5]. While ATR seems to specifically recognize single-stranded DNA generated during DNA replication fork stalling or resection during HR[6,7], ATM and DNA-PKcs are the PIKKs that respond specifically to DSBs. DNA-PKcs is a target for ATM in this response[5,8]. However, ATM- and DNA-PKcs-dependent DNA repair mechanisms are often presented as independent. Immediately after DSB-mediated activation, ATM is able to promote efficient DSB-signal amplification, phosphorylate proteins involved in cell cycle checkpoints, activate DNA repair and induce apoptosis if required. In addition, ATM phosphorylates the histone H2AX that, together with several repair and DNA damage-signalling factors accumulate at the DSB[9]. DNA-PKcs has limited functions at these particular levels. Even though DNA-PKcs can participate in DSB signalling through H2AX phosphorylation (reviewed in[10]), or p53 activation and cell-cycle arrest - by regulating the G2/M checkpoint [11,12]-, its function is dispensable when ATM is present[13-16]. In relation to DSB signalling, DNA-PKcs can phosphorylate H2AX in the absence of ATM but not in its presence, and not even in the presence of kinase-inactive ATM, which suggests that ATM physically blocks DNA-PKcs from accessing H2AX (reviewed in[10]). It has been proposed that DNA-PK plays an anti-apoptotic role that could suppress a p53-independent apoptosis response[17]. This function would be contrary to that carried out by ATM, and could thus balance its effects. Therefore, although ATM and DNA-PKcs may have partially overlapping functions in DSB signalling, p53 activation and cell-cycle arrest, the current knowledge of the functions of DNA-PKcs at this level restricts it to being a back-up kinase.

DSB repair: one step further

While ATM is the master regulatory kinase in the DDR in response to DSBs, no specific mechanistic or structural function during DSB repair itself has yet been attributed to this kinase. ATM deficiency results in a chromosome instability syndrome due to incomplete DSB repair and persistence of some residual DSBs. The immunodeficiency and lymphoid malignancies

characteristic of the ataxia-telangiectasia (AT) syndrome can mainly be explained by the accumulation of unrepaired breaks that may eventually be involved in illegitimate repair events and give rise to chromosome rearrangements such as translocations. In turn, DNA-PKcs displays a well-known mechanistic and regulatory role during NHEJ-mediated DSB repair[18-20]. Faced with DSBs, DNA-PKcs is quickly recruited to the broken ends by Ku [21,22] and helps tether DNA ends together. After autophosphorylation, DNA-PKcs is paradoxically released from the DNA ends to allow efficient end-joining[23-25]. Mutations in the *PRKDC* gene that result in truncated DNA-PKcs proteins that lack kinase activity have been described in mice [26,27], dogs [28] and horses[29]. These mutations translate into a severe combined immunodeficiency (SCID) phenotype due to V(D)J recombination impairment, resulting in B and T lymphocyte development defects. The total absence of the kinase DNA-PKcs is probably incompatible with human life because only two cases of a deletion and a mutation in the *PRKDC* gene respectively have been described to date [30,31]. These alterations only led to a partial defect in the DNA-PKcs function that most probably explains the SCID phenotype of the affected individuals. Nonetheless, animal [32] and human cell lines which completely lack DNA-PKcs protein have been artificially generated, and they all present proliferation and genome stability deficits such as diverse chromosome aberrations [33-35] or an increased frequency of gene amplification events [36,37], which are thought to be the result of illegitimate DSB repair[38]. Moreover, after irradiation, DNA-PKcs deficient cells accumulate an even higher proportion of chromosome rearrangements[33,39], indicative of a DNA repair defect that mainly results in DSB breaks being rejoined unfaithfully. Thus, the absence of either ATM or DNA-PKcs leads to an abnormal persistence of DSBs and to the accumulation of illegitimate joining events that threaten genomic stability.

Several lines of evidence strongly argue for cross-talk between ATM and DNA-PKcs in DSB repair. First, the combined deficiency of both ATM and DNA-PKcs leads to synthetic lethality in mice embryos[40]. Second, ATM and DNA-PKcs are able to phosphorylate many common targets

required for DSB repair *in vivo* and/or *in vitro*, including most components of the classical NHEJ pathway, such as Ku70, Ku80, DNA Ligase IV, XRCC4, XLF and Artemis[21,41-45], although no clear function has yet been elucidated for these phosphorylation events in DSB repair. Third, ATM and DNA-PKcs do not only share common targets involved in DSB repair, but they also seem to modulate their respective repair activities. ATM is able to phosphorylate DNA-PKcs at the Thr2609 cluster to stimulate endonucleolytic activity and the proper processing of otherwise non-ligatable DNA ends, which is a critical step in the correct repair function of DNA-PKcs[8].Moreover, DNA-PKcs may be able to transcriptionally regulate ATM as DNA-PKcs deficiency results in down-regulation of ATM[46].

Although the relationship between ATM and DNA-PKcs in achieving complete or legitimate DSB repair has been described as overlapping, complementary or synergistic, it still needs to be clearly defined. Rather than making a detailed list of the activators and substrates of each kinase, we attempt to compare the nature of the repair defects caused by the absence of ATM and DNA-PKcs that lead to unfaithful DSB repair, and thus contribute to the onset of genomic instability. The reviewed studies show that, rather than overlapping, the roles of ATM and DNA-PKcs in DSB repair can be described as complementary, since the presence of the two kinases is absolutely necessary in order to avoid faulty DSB rejoining and achieve legitimate repair.

Complete, albeit slow DSB repair in DNA-PKcs-deficient cells

Analyses of DSB rejoining kinetics using field inversion gel electrophoresis (FIGE) techniques have been widely carried out to study different NHEJ-mutants. In all cell types analysed so far, DSB repair follows biphasic repair kinetics, with a fast repair phase followed by a slow repair phase. Normal cells repair most of the inflicted damage in the fast repair phase during the very early post-irradiation times. The absence of DNA-PKcs kinase always results in a severe delay in the fast repair component (cells derived from *SCID mice*:[47 ,48]; *M059J cells*:[49 ,50 ,51]; *DNA-PKcs*$^{-/-}$

mouse embryonic fibroblasts:[39]). Thus, while normal cells rejoin most of the radio-induced DSBs during the first two hours after irradiation and reach the slow repair phase bearing a low number of DSBs (20-30%), DNA-PKcs-deficient cells reach this phase with a significantly higher number of unrejoined breaks (50-60%)[51].

As DNA-PKcs-deficient cells lack one of the main components of the NHEJ repair machinery, we could expect that irradiation of these cells would translate into a massive accumulation of residual DSBs, because of an inability to rejoin radio-induced DNA broken ends. Instead, the acute radiosensitivity of DNA-PKcs-deficient cells translates into a sharp increase in exchange type rearrangements as well as complex aberrations[33,34,39,52]. As stated before, DNA-PKcs cells retain rejoining ability and results from previous works strongly suggest that the confluence of multiple breaks increases the probabilities of illegitimate rejoining[53]. Thus, the sharp increase in exchange type rearrangements in irradiated DNA-PKcs cells can be explained because of the likelihood of the slowly repaired DSBs to be misrejoined[53,54]. Despite impairment in the fast component of repair, the overall joining ability of DNA-PKcs-defective cells is not abolished. Instead, DNA-PKcs defective cells are able to repair these unrejoined breaks during the slow repair phase, reaching complete repair 48 hours after IR exposure, a far longer time than that taken by normal cells (24h). Thus, the NHEJ repair pathway is crucial for fast repair to take place. Specifically, DNA-PKcs deficiency implies a repair defect based on slow DSBs-rejoining kinetics, resulting in delayed repair and the accumulation of DSBs prone to misrejoining.

Fast, but incomplete DSB repair in ATM-deficient cells

ATM-deficient cells fail to arrest in G1 partly due to the impaired activation of p53[55]. ATM mutant cells exhibit radio-resistant DNA synthesis in S phase and continue to synthesize DNA following exposure to IR, but they also fail to arrest in G2. This incorrect checkpoint functioning allows DNA damage to progress through the cell cycle, favouring the persistence of unrepaired

chromosome breaks in the following M phase[56-59]. Nevertheless, faulty repair is not only explained by checkpoint arrest failure. Years ago, Cornforth and Bedford showed that ATM deficient cells accumulate a significantly higher frequency of residual breaks, even when these cells were prevented from cycling by applying the premature chromosome condensation technique[60]. Related to these cytogenetic results, the very same conclusions have been reached in analyses of DSB repair kinetics in ATM-deficient cells using FIGE as well as by measuring the rate of γH2AX immunofluorescence foci loss. Contrary to DNA-PKcs deficient cells, AT and normal cells initially follow the same DSB joining kinetics, and are able to effectively rejoin 70-80% of the breaks with fast kinetics. However, while normal cells rejoin the rest of the breaks (20-30%) with slow kinetics and reach complete repair 24h later, AT cells maintain a residual level of unrepaired breaks for a long time after irradiation[61-63]. Thus, while DNA-PKcs cells display slower rejoining kinetics but eventually reach complete repair, ATM deficient cells display normal repair kinetics for most DSBs but repair is ultimately incomplete. Residual breaks represent approximately 10-15% of the radio-induced breaks in ATM-deficient cells, and this fraction continues to be unrejoined at 48h after IR exposure, or even longer[64,65]. This repair defect favours the propagation of residual breaks, some of which can be identified as unrepaired chromosome breaks not only in the next but also in subsequent mitosis[60,66,67]. In a recent study we checked whether visible residual breaks in mitotic chromosomes from irradiated ATM-deficient cells displayed proper signalling of DNA repair factors. We reported that a significant fraction of unrepaired chromosome breaks lacked γH2AX and Mre11 signalling in AT cells[63], and were therefore invisible to the DDR machinery. If these breaks are not efficiently sensed they can accumulate in an unrepaired state through subsequent cell divisions, which accounts for the higher proportion of residual breaks described in AT cells. To summarize the above described works, the repair defect of ATM-deficient cells can be best explained as a defect in DNA break processing with the persistence of some DSBs.

Characteristics of the residual DSBs in ATM-deficient cells

There has been extensive research into characterizing the nature of residual breaks in AT cells. Some years ago Jeggo's laboratory proposed that unrepaired residual breaks could correspond to complex lesions that were refractory to NHEJ due to faulty ATM-dependent activation of Artemis nuclease[65,68]. This model was proposed because the loss of either ATM or Artemis, an end-processing nuclease required for proper DSB resolution during V(D)J[44,69,70], led to identical DSB repair defects. It was proposed that Artemis was activated by ATM after cell-irradiation[65]; however, this model was discarded after it was demonstrated that Artemis activation depends solely on DNA-PKcs kinase, and not on ATM[71-73]. More recently, the same research group proposed an alternative model that identifies ATM-dependent residual breaks as those induced close to or within heterochromatin[74]. The authors showed that knockdown of KAP-1, a protein that triggers heterochromatin formation via interaction with other proteins, alleviates the requirement for ATM in DSB repair. According to this model, ATM-mediated phosphorylation of KAP-1 (a core component of heterochromatin) would be a crucial step for relaxing heterochromatin regions that otherwise would be inaccessible, thus facilitating its repair. [74]. Curiously, no cytogenetic studies published to date with human AT cells from affected individuals have reported increased frequency of residual chromosome breaks or chromosome rearrangements involving breakpoints corresponding to defined heterochromatic regions, such as those regions in chromosomes 1, 9 and 16 or pericentromeric regions of human chromosomes[75-79], suggesting that ATM roles in DSB repair are heterogeneous and keep on demanding continuous research.

In this regard,, evidence suggests that this kinase is indeed involved in the resolution of breaks located in more accessible regions, for example those arising during V(D)J or CSR. The creation of many diverse lymphocyte receptors to identify potential pathogens has evolved by breaking and randomly re-sorting the gene segments coding for antigen receptors. Like spontaneous or radiation-induced DSBs, the programmed DSBs produced in lymphocytes during V(D)J or CSR recombination events do activate specific components of the DDR pathway and are also repaired

via NHEJ (reviewed in[80]). During V(D)J recombination, two types of DNA ends are generated after DNA resection: two signal ends and two coding ends. Both signal ends present blunt terminations that are easily joined together by the NHEJ machinery without further processing, creating a signal joint. On the contrary, both coding ends present a hairpin structure at their ends that must be opened and processed in a DNA-PKcs- and Artemis-dependent manner before joining them together to create a coding joint. CSR events consist of the breaking of switch regions and subsequent rejoining in an NHEJ-dependent way, but no hairpin structure is formed during this process. The absence of DNA-PKcs leads to some defects in CSR [81,82] and severely compromises coding joint formation during V(D)J[32]. Furthermore, and although repair of DSBs arising during these processes is dependent on NHEJ, the ATM kinase also plays a role in these mechanisms. Although ATM deficiency does not result in a profound block in lymphocyte development, the fidelity of V(D)J recombination is eventually affected in the absence of ATM[83,84]. As a result, blood lymphocytes from AT patients show a high incidence of chromosomal rearrangements that involve the lymphocyte antigen receptor loci[79,85]. Thus, interestingly, studies on CSR and V(D)J-dependent DSB resolution have provided an additional way of determining not only the roles of ATM and DNA-PKcs in DSB repair, but also their interactions during DSB resolution.

Recombination events help reveal a connection between ATM and DNA-PKcs in NHEJ

Similarly to the results of the above described cytogenetic and DSB repair kinetics studies, works with ATM deficient cells stimulated to undergo V(D)J or CSR also show that these cells accumulate residual DSBs that eventually misrejoin and translate into rearranged chromosomes. B cells from *Atm*$^{-/-}$ mice stimulated to undergo CSR accumulate IgH-specific chromosome breaks, display an increase in c-myc/IgH translocations and present general instability outside of the IgH locus[86,87]. Studies on V(D)J recombination have yielded very similar results: B lymphocytes from *Atm*$^{-/-}$ mice were reported to display breaks generated during V(D)J recombination that

persisted in an unrepaired state for even weeks[88]. In these lymphocytes, unresolved coding ends accumulated despite the presence of a fully functional NHEJ pathway, thus becoming potential substrates for future translocations. Interestingly, in *Atm*$^{-/-}$ cells the hairpin structure of the coding ends – whose resolution depends on DNA-PKcs – was correctly and faithfully processed. However, many of these ends were later involved in the formation of hybrid joints between a coding and a signal end[89,90]. This structure highly resembles misrepair events such as chromosomal translocations that are also due to illegitimate joining. All these studies suggest that the NHEJ pathway is not completely efficient or accurate in the absence of the ATM protein, whose presence seems to ensure legitimate joining of the correct ends. In this sense, the authors suggested that ATM may stabilize recombination DSB intermediates during chromosomal V(D)J recombination, or it may facilitate DNA end-joining and prevent broken DNA ends from participating in chromosome deletions, inversions and translocations[86,88-90].

In order to try to define the interplay between ATM kinase and the NHEJ machinery, simultaneous DNA-PKcs and ATM deficiency during V(D)J recombination was evaluated. The combined deficiency of both kinases leads to the accumulation of unrejoined signal ends and to the lack of signal joint fidelity[91,92]. In these cells, signal ends were frequently resolved in an aberrant way, resulting in potentially dangerous chromosomal translocations and deletions[91,92].The authors highlight that the kinase activity of both proteins is critical for their function in properly resolving DSBs generated during V(D)J recombination events, and propose that these proteins phosphorylate common substrates that participate in this process. Similarly, CSR recombination was evaluated in *Atm*$^{-/-}$ B cells in which DNA-PKcs activity was depleted. The results show a severe defect in DNA repair that translates into a synergistic increase in chromosomal aberrations. These results suggest that the two kinases act in coordination to repair programmed DSBs arising during CSR[93]. The authors propose an attractive model in which after DNA-PKcs fails to repair these DSBs quickly, DNA-PKcs is phosphorylated and released from the break in an ATM-dependent manner, while at the same time cell-cycle arrest, apoptosis or delayed repair are triggered[93]. This model is similar

to that proposed by Shrivastav *et al*. in which, given the mutual regulation of their activities in relation to DSB repair, ATM and DNA-PKcs would be able to activate and stabilize each other so that, the kinases themselves and their respective substrates, could reach the DSB in an orderly way and cooperate alternatively in effective DSB repair[94].

All together there is clear evidence that both ATM and DNA-PKcs are necessary for achieving proper DSB repair in general, and correct V(D)J- and CSR-intermediate DSB resolution in particular. Although in these recombination events the final DSB resolution depends on a fully functional NHEJ pathway that ensures complete repair, evidence is provided that ATM is also necessary in order to avoid the persistence of residual breaks and to favour end-joining of the correct DNA ends.

ATM and DNA-PKcs play complementary roles in DSB repair

Classical cytogenetic studies and DSB rejoining kinetics studies performed with ATM-deficient cells describe that the absence of ATM translates into the persistence of residual breaks that can remain unrepaired or be eventually involved in the formation of translocations or dicentrics. In turn, breaks arising in DNA-PKcs-deficient cells are repaired very slowly, which favours incorrect ends being joined. Recent literature on the repair of radiation-induced or physiologically formed DNA breaks strongly suggests that ATM and DNA-PKcs may indeed carry out complementary activities during DSB repair. Studies show that NHEJ predominates to repair most DSBs in mammalian cells [95] and that Ku heterodimer is targeted to the sites of damage within minutes[96], where it surrounds the DSB and from where it recruits DNA-PKcs protein to the break. In parallel, the MRN complex is also involved in the initial processing of DSBs. After early positioning to the break site, MRN recruits ATM[97], and ATM-dependent DNA-PKcs phosphorylation may stimulate proper processing of DNA ends, as this interaction is crucial for DNA repair to take place[8]. This model implies that both kinases are involved in break rejoining, and that their interaction at the DSB is

necessary to achieve fast and complete DSB repair. The presence of DNA-PKcs guarantees that most breaks will join in a timely manner[33,39,51], while the presence of the ATM protein ensures the complete repair of DSBs by stabilizing them at DNA repair complexes and/or favouring the joining of correct DNA ends [39,54,90,93] (Figure 1). As there is evidence that a single misrejoining event can lead to the onset of a malignancy, it is imperative that DSB repair mechanisms are always able and ready to carry out their functions accurately. DSB repair must be fast and complete to avoid open ends from accumulating, which would increase the probability of eventual illegitimate rejoining. Thus, both ATM and DNA-PKcs are indispensable for ensuring the fidelity of the repair process. Further research into the variations in the levels or functions of either kinase, depending on the cell-cycle status, the cell type, the chromatin status and the type of damage inflicted, is necessary for clarifying further ATM and DNA-PKcs cross-talk actions in DSB repair.

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Figure 1

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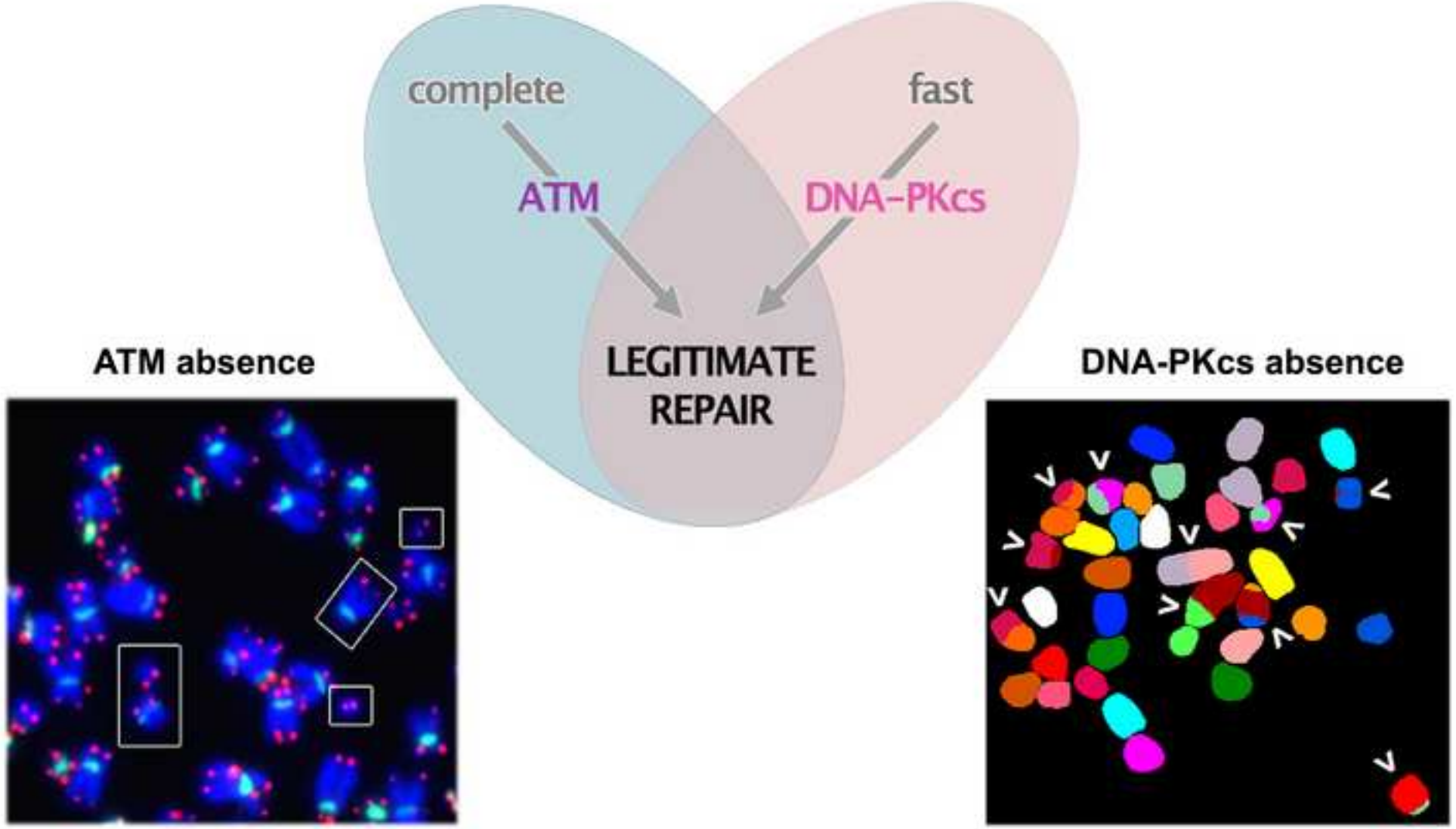

Figure caption

Figure 1. ATM and DNA-PKcs play complementary roles in DSB repair. While ATM ensures complete repair, the DNA-PKcs protein guarantees fast DSB joining kinetics. Both fast and complete repair are necessary to achieve legitimate repair. **ATM absence**. The image shows a metaphase of irradiated AT cells after centromeric (green) and telomeric (red) FISH (Fluorescence *in situ* hybridization). Initially the AT cells display a normal repair speed; however, they do not ultimately complete repair. As a consequence, AT cells accumulate unrepaired DSBs that can eventually be identified as broken chromosomes in which a single telomere pair is present (white squares). **DNA-PKcs absence**. The image shows a metaphase of irradiated DNA-PKcs deficient cells after SKY (Spectral karyotyping), which allows simultaneous visualization of all the chromosome pairs. DNA-PKcs-deficient cells ultimately reach complete repair, albeit with slow kinetics. The delayed DSB joining kinetics favour the joining of incorrect ends, which translates into the accumulation of exchange type aberrations such as translocations, dicentrics and insertions (arrow heads). Therefore, the presence and proper functioning of both kinases are necessary to ensure the fidelity of the repair process.